

Up-regulation of system A activity in the regenerating rat liver

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System A activity for neutral amino acid transport, measured as the MeAIB-sensitive Na^+ -dependent L-alanine uptake, is induced 6 h after partial hepatectomy in plasma membrane vesicles from rat livers. Other Na^+ -dependent transporters, like system ASC (MeAIB-insensitive Na^+ -dependent L-alanine transport) and the nucleoside carrier show similar inductions. Up-regulation of system A is not explained by changes in the dissipation rate of the Na^+ transmembrane gradient, as deduced from uptake measurements performed in the presence of monensin. To determine whether induced system A shared any similarity with the activity found in hepatoma cell lines, we analyzed the *N*-ethylmaleimide (NEM) sensitivity of system A in both regenerating and control rat liver plasma membrane vesicles. NEM treatment was equally effective in inhibiting system A in both experimental groups. Thus, during the prereplicative phase of liver growth, a transport activity similar to basal system A is up-regulated in liver parenchymal cells, by a stable mechanism that does not involve changes in the Na^+ transmembrane gradient.

Amino acid transport; System A; Liver; Regeneration

1. INTRODUCTION

System A is the major transport system involved in concentrative neutral amino acid transport in most mammalian cells. It is regulated *in vivo* and *in vitro* by both hormonal and nutritional factors [1–3]. System A expression is also regulated at the transcriptional level in cultured cells by amino acid availability, an effect which is called adaptive regulation and involves the de-repression of system A by a putative regulatory gene [4,5]. Also, anisotonic shock seems to selectively induce system A by protein synthesis-dependent mechanisms which seem to occur through pathways other than the ones involved in adaptive regulation [6,7]. Other evidence suggests that system A activity is enhanced in transformed cells as well as in cells induced to proliferate or differentiate [3,8–10]. Liver system A activity is induced in different physiological conditions associated with liver hypertrophy and/or hyperplasia, like pregnancy, development or genetic obesity [11–13]. Hepatoma cell lines also express system A activity, although the sensitivity of the carrier to some sulfhydryl-modifying reagents is markedly different to the one found in cultured hepatocytes [14]. Interestingly, the activity of an uncharacterized high-affinity component of transport that was attributable to system A is also induced in rat hepatocytes isolated from regenerating livers, and it has even been suggested that induction of

system A may be a permissive factor in liver proliferation after partial hepatectomy [15]. The biochemical basis of this likely up-regulation is not well understood. In this way we wanted firstly to ascertain whether the induction of system A is a stable phenomenon that can be retained in plasma membrane vesicles from regenerating rat livers; secondly to study to what extent the induction is dependent on the Na^+ transmembrane gradient; and, finally, to analyze the sensitivity of the induced activity to *N*-ethylmaleimide (NEM) as a way to determine whether or not this system shares similarities with the one present in some transformed liver cells.

2. MATERIALS AND METHODS

2.1. Animals and surgery

Overnight fasted male Wistar rats weighing 200–230 g were used. They were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg b.wt.) and immediately laparotomized. A 70% hepatectomy was then performed as previously described [16]. Another set of animals did not undergo hepatectomy but only liver extrusion, and they were used as sham-operated controls. All the surgical procedures were performed at the beginning of the light cycle (08:00 h).

2.2. Isolation of liver plasma membrane vesicles

The liver lobes excised were used for plasma membrane preparation as non-operated controls and were called C0. 6 h after surgery, both hepatectomized and sham-operated rats were killed by decapitation and the livers immediately excised for plasma membrane isolation: those samples were called R6 and C6, respectively. For every R6 preparation two livers were pooled. The method used to partially purify plasma membrane vesicles from rat liver was the one reported by [17], as modified by [11]. This technique has been validated in our laboratory and yields considerable amounts of plasma membrane vesicles that retain active transport systems for neutral amino acids [11,12,18], monocarboxylic acids [19] and nucleosides [20]. Furthermore, as we recently reported, plasma membrane fractions from both

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hepatectomized and sham-operated rats have similar apparent volumes and show identical protein profiles when run on SDS-PAGE [21]. This suggests that both preparations are similar from a physicochemical point of view.

2.3. Transport measurements

Uptake rates were determined by a filtration procedure, as previously described [11]. Briefly, plasma membrane vesicles were mixed with transport media to give the following final concentrations: 0.25 M sucrose, 0.2 mM CaCl_2 , 10 mM MgCl_2 , 10 mM HEPES/KOH (pH 7.4), either Na^+ or K^+ salts (both at 100 mM) and radiolabeled substrates at the indicated concentrations. The reaction was stopped at the indicated times by diluting the mixture up to 1 ml with an ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, 0.2 mM CaCl_2 , 10 mM HEPES/KOH at pH 7.4). Then, the content of the tube was quickly filtered through a nitrocellulose membrane (0.45 μm pore size) (Schleicher and Schuell, Germany) and immediately washed with 4 ml of the ice-cold stop solution. System A activity was analysed as previously reported [7,13], as the fraction of Na^+ -dependent L-alanine uptake inhibited by an excess of MeAIB. Radiolabeled substrates were L-[2,3- ^3H]alanine, L-[4,5- ^3H]leucine, D-[1- ^3H]glucose (Amersham, Bucks, UK) and [5,6- ^3H]uridine (NEN, Boston, MA). The specific radioactivities of the different substrates in the incubation media were 45 $\mu\text{Ci}/\mu\text{mol}$ for L-alanine, 90 $\mu\text{Ci}/\mu\text{mol}$ for L-leucine, 7 $\mu\text{Ci}/\mu\text{mol}$ for D-glucose and 9 $\mu\text{Ci}/\mu\text{mol}$ for uridine. In the kinetic studies of L-alanine transport, the specific radioactivity of the amino acid decreased from 90 $\mu\text{Ci}/\mu\text{mol}$ to 5 $\mu\text{Ci}/\mu\text{mol}$ as the substrate concentration increased. In all cases the sensitivity of the measurements was high. Regular countings for 0.25 mM L-alanine uptake were around 4,000 dpm and 1,000 dpm for NaSCN and KSCN media, respectively. Blank values were below 200 dpm and always subtracted from the uptake measurements.

The studies using monensin were performed as previously described [21] by incubating, simultaneously, the ionophore at a concentration of 20 μM and the substrate, L-alanine, at 0.25 mM. Since dilution of the ionophore required 2% DMSO, uptake rates in its absence were measured in a DMSO-supplemented medium.

2.4. Effect of N-ethylmaleimide on Na^+ -dependent L-alanine uptake

The effect of NEM on Na^+ -dependent L-alanine uptake was studied as previously described [14]. Liver plasma membrane vesicles were preincubated with increasing concentrations of NEM (0.625–5 mM) for 30 min at 25°C. In order to wash off the inhibitor, vesicles either treated or not with NEM were sedimented by high-speed centrifugation using a microfuge and then the pellets were resuspended in the same medium in which the vesicles had been purified. The resulting suspension was used for transport measurements, as indicated above, and for protein content determination. Non-treated controls run in parallel with the NEM-treated samples were used to determine whether vesicle manipulation by itself could induce any change in basal transport activities. All the uptake measurements were done at a concentration of L-alanine of 0.25 mM. As we show in Table I (see section 3), at this substrate concentration system A activity is responsible for 80% of the total Na^+ -dependent L-alanine uptake.

3. RESULTS

3.1. Na^+ -dependent L-alanine uptake

L-Alanine uptake, when measured in a NaSCN medium as a function of the incubation time, was clearly concentrative (not shown). Preparations from hepatectomized rats showed a higher overshoot over the equilibrium levels than those from sham-operated animals. A 3 s incubation gave uptake values close to initial velocity and, thus, this incubation time was used in all the other experiments. The concentration dependence

of Na^+ -dependent L-alanine uptake was studied for substrate concentrations ranging from 0.1 to 20 mM. Woolf–Augustinson–Hofstee plots of the experimental data are shown in Fig. 1. For all the concentrations tested, Na^+ -dependent uptake was higher in R6 than in C6. In all the experiments so far performed, no differences between C6 and C0 controls have been found (not shown). The difference between R6 and C6 preparations was much higher at low substrate concentrations, which is in agreement with the selective induction of a high-affinity component of transport reported by [22] when using isolated rat hepatocytes and amino acid analogues as substrates. Nevertheless, when we tried to fit the transport data according to a non-linear regression analysis using the Enzfitter software (Elsevier, Biosoft, Cambridge, UK), we found that the best fit of the Na^+ -dependent L-alanine uptake resulted in a single saturable agency with kinetic parameters as follows: V_{max} , 1.3 and 1.5 pmol/3 s/ μg protein for C6 and R6 preparations, respectively; K_m , 3 and 1.7 mM for C6 and R6 preparations, respectively.

3.2. MeAIB-sensitive and insensitive Na^+ -dependent L-alanine uptake

To determine to what extent the induction of Na^+ -dependent L-alanine uptake in R6 was only attributable to system A, we performed a series of experiments where Na^+ -dependent L-alanine uptake was measured either in the presence or the absence of saturating concentrations of MeAIB. Results are shown in Table I.

Table I

Effect of partial hepatectomy on Me-AIB sensitive and insensitive Na^+ -dependent L-alanine uptake

[L-Alanine] mM	L-alanine uptake (pmol/3 s · mg protein)			
	Me-AIB sensitive (A)		Me-AIB insensitive (ASC)	
	C6	R6	C6	R6
0.1	38 ± 4	92 ± 9***	11 ± 3	11 ± 3
0.25	62 ± 11	154 ± 29**	17 ± 3	32 ± 4*
0.5	84 ± 18	212 ± 34**	66 ± 14	118 ± 16*
1	289 ± 15	447 ± 52**	67 ± 16	191 ± 36**
2	318 ± 38	545 ± 92	153 ± 40	201 ± 43

Plasma membrane vesicles were purified either from regenerating rat livers, 6 h after partial hepatectomy (R6), or from their sham-operated controls (C6). Vesicles were incubated in the presence of increasing concentrations of L-alanine, either in a NaSCN or a KSCN medium. To determine system A activity, saturating concentrations of MeAIB were used (20- to 200-fold greater than substrate concentrations). Results correspond to the MeAIB-sensitive and -insensitive fraction of total Na^+ -dependent L-alanine uptake and are the mean ± S.E.M. of 6–11 determinations on pooled plasma membrane fractions from at least three independent preparations. Na^+ -dependent transport was measured by subtracting uptake rates measured in a KSCN medium from those measured in a NaSCN medium. Statistical comparisons were done using from Student's *t*-test (* P < 0.05, ** P < 0.01, *** P < 0.001).

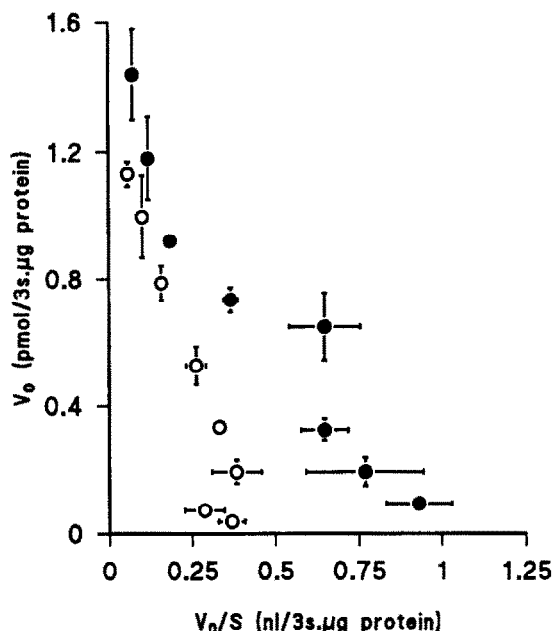


Fig. 1. Woolf-Augustinsson-Hofstee plot of Na^+ -dependent L-alanine uptake. Na^+ -dependent uptake was calculated by subtracting transport rates measured in a KSCN medium from those determined in a NaSCN medium. Substrate concentrations ranged from 0.1 to 20 mM. R6 and C6 preparations are shown as filled and open circles, respectively. Results are the mean of triplicate estimates made on 4–6 independent preparations.

For all the concentrations tested, transport through system A was responsible for most of the total Na^+ -dependent L-alanine uptake induction. However, MeAIB-resistant L-alanine transport (assumed to be system ASC) was also enhanced in R6 when compared to C6 preparations, although transport through system A was always much higher (2- to 8-fold, depending on the concentration tested) than the one attributable to system ASC.

3.3. Effect of partial hepatectomy on other transport systems

To ascertain whether the induction of system A and, to a less extent, system ASC was rather specific or just the consequence of a general tendency to increase the activities of plasma membrane transporters, we determined the uptake rates of other solutes. Results are shown in Table II. Uridine transport was markedly enhanced in R6 fractions when compared to their sham-operated controls. This increase is in agreement with previous observations from our group [21]. Leucine transport which preferentially occurs via system L, a Na^+ -independent carrier, was not significantly modified by partial hepatectomy; neither was glucose transport. Thus, the adaptive response to hepatectomy seemed to involve, at least, three separate Na^+ -dependent transport systems, A, ASC, and the recently characterized liver nucleoside transporter.

3.4. Effect of monensin on system A induction

To determine whether up-regulation of system A could be explained by a different dissipation rate of the Na^+ transmembrane gradient, we performed another series of experiments where vesicles were incubated, as indicated above, either in the presence or the absence of monensin, following [21]. Results are shown in Table III. Monensin did not affect 0.25 mM L-alanine uptake in a KSCN medium, although those rates, attributable to simple diffusion, were higher in R6 than in C6 preparations. As expected, the addition of the ionophore decreased Na^+ -dependent L-alanine uptake, although R6 fractions still showed a 2-fold induction of transport over C6. The totality of this enhancement was attributable to the remaining system A activity.

3.5. Effect of NEM on Na^+ -dependent L-alanine uptake

The sensitivity of C6 preparations to increasing concentrations of NEM is shown in Fig. 2. NEM treatment did not modify L-alanine uptake in a KSCN medium, but strongly depleted Na^+ -dependent transport. Total inhibition was achieved at 5 mM NEM and then, another series of experiments was performed using this concentration of inhibitor (Table IV). Vesicle manipulation induced a significant decrease in Na^+ -dependent L-alanine uptake, which was similar in terms of percentage in both C6 and R6 preparations (about 40–45% lower than the original values). When vesicles had been previously treated with 5 mM NEM, most of the Na^+ -dependent activity disappeared, and indeed only a slight but not significant resistance to NEM was found in R6 fractions.

Table II

Solute uptake rates by plasma membrane vesicles from regenerating rat livers

	Physiological situation	
	C6	R6
0.25 mM L-alanine uptake (pmol/3 s · mg protein)	72 ± 15 (4)	192 ± 44* (3)
5 µM uridine uptake (pmol/3 s · mg protein)	0.69 ± 0.05 (3)	1.82 ± 0.11*** (3)
0.2 mM L-leucine uptake (pmol/3 s · mg protein)	55 ± 8 (8)	55 ± 2 (6)
2 mM D-glucose uptake (pmol/3 s · mg protein)	440 ± 80 (8)	550 ± 50 (8)

Plasma membrane vesicles from regenerating rat livers (R6) and their sham-operated controls (C6) were incubated, as indicated in section 2, in the presence of selected solutes (L-Ala, L-Leu, uridine and D-gluc). L-Alanine and uridine data correspond to the Na^+ -dependent uptake rates, calculated as the difference between carrier-mediated and non-carrier-mediated transport. L-Leucine and D-glucose transport were exactly the same either in a KSCN or a NaSCN medium and also involved both saturating and non-saturating agencies. The number of observations made on pooled fractions from at least three independent preparations are given in parentheses. Statistical comparisons were done by Student's *t*-test (* P < 0.05; *** P < 0.001).

4. DISCUSSION

This study shows that different Na^+ -dependent transport systems are up-regulated after partial hepatectomy, while other Na^+ -independent transporters are not. The apparent induction of system ASC should be considered of minor relevance because, at physiological L-alanine concentrations, only a 10–35% of the total Na^+ -dependent uptake occurs via the MeAIB-insensitive component of transport. Thus, system A is the main agency responsible for neutral amino acid uptake in the regenerating rat liver early after hepatectomy. Up-regulation of system A activity results in a lower apparent K_m of the Na^+ -dependent L-alanine uptake. Indeed, L-alanine uptake rates were much higher in R6 than in C6 preparations when measured in the range of physiological concentrations of the amino acid. A previous report showed a similar response in isolated hepatocytes from hepatectomized rats, although it was accounted for by an unidentified agency showing high affinity for its substrate, the non-metabolizable analogue α -aminoisobutyric acid (AIB) [22].

Leffert and co-workers [15] had previously suggested that system A up-regulation could be a key factor in the development of the cellular mechanisms responsible for the entry into S phase of quiescent hepatocytes induced to proliferate after partial hepatectomy. The induction of Na^+ -dependent transport systems like system A could be attributable to an altered transmembrane electrical gradient. Indeed, changes in membrane polarization

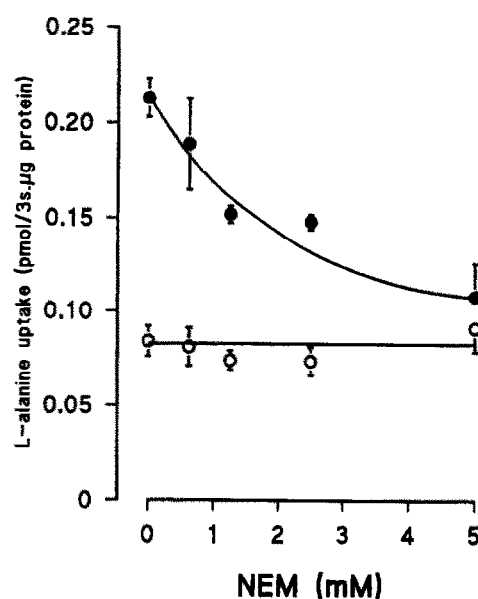


Fig. 2. Effect of NEM treatment on L-alanine uptake into plasma membrane vesicles from rat liver. Plasma membrane vesicles from sham-operated rats (C6) were preincubated, as indicated in section 2, in the presence of increasing concentrations of NEM, either in a NaSCN (●) or a KSCN medium (○). Transport of 0.25 mM L-alanine was then monitored in triplicate, using pools of plasma membrane vesicles from four independent preparations. Results are the mean \pm S.E.M.

may play a key role in the initiation of liver growth (for review see [15,23]). Enhanced activity of the amiloride-sensitive Na^+ carrier was described many years ago [24] as a necessary process to initiate hepatocyte proliferation. Peptides involved in liver regeneration, like glucagon and epidermal growth factor (EGF), induce Na^+ -dependent amino acid transport by short-term mechanisms, not involving protein synthesis, and exclusively dependent on membrane hyperpolarization [25,26]. We now show the effect is stable enough to be present in

Table III

Effect of monensin on L-alanine uptake

Incubation medium	L-alanine uptake (pmol/3 s · mg protein)	
	C6	R6
NaSCN	98 \pm 4	206 \pm 17***
NaSCN + 20 μ M monensin	77 \pm 8	134 \pm 8***
KSCN	39 \pm 3	61 \pm 5**
KSCN + 20 μ M monensin	46 \pm 4	68 \pm 2**
NaSCN \pm MeAIB	50 \pm 6	71 \pm 8
NaSCN + MeAIB + 20 μ M monensin	41 \pm 4	56 \pm 7
Na^+ -dependent uptake		
–monensin	59	145
+monensin	31	66
System A activity		
–monensin	48	135
+monensin	36	78

Vesicles from both experimental groups, C6 and R6, were incubated, as indicated in section 2, either in a NaSCN or a KSCN medium, with or without monensin, and in the presence of 25 mM MeAIB, to selectively block L-alanine uptake through system A. L-Alanine was used at 0.25 mM. The results are the mean \pm S.E.M. of 4–12 separate estimates made on pooled plasma membrane fractions from at least three independent preparations. Statistical comparisons were done using Student's *t*-test (** P < 0.01, *** P < 0.001).

Table IV

Effect of NEM treatment on Na^+ -dependent L-alanine uptake in plasma membrane vesicles from regenerating rat livers

	L-Alanine uptake (pmol/3 s · mg protein)	
	C6	R6
NaSCN	73 \pm 4	^b 127 \pm 6***
KSCN	32 \pm 5	43 \pm 5
NaSCN + NEM	^a 51 \pm 7*	^b 62 \pm 7***
KSCN + NEM	47 \pm 7	41 \pm 8

C6 and R6 preparations were preincubated with 5 mM NEM, as indicated in the Experimental section, and 0.25 mM L-alanine uptake was monitored either in a NaSCN or a KSCN medium. The effects induced by NEM treatment (^a) and partial hepatectomy (^b) were assessed using the Student's *t*-test (* P < 0.05; *** P < 0.001). The results are the mean \pm S.E.M. of 6 separate observations made on pooled fractions from at least three independent preparations.

preparations of liver plasma membrane vesicles and does not depend on a different dissipation rate of the Na^+ transmembrane gradient. This finding is in agreement with the reported increase of Na^+ , K^+ -ATPase β_1 mRNA levels early after partial hepatectomy [27] and our previous observation on the Na^+ -dependent uridine transporter [21], suggesting that Na^+ -coupled transport during liver regeneration is increased by protein synthesis-dependent mechanisms. A selective synthesis of plasma membrane proteins is also in agreement with a different glycoprotein pattern of plasma membranes from regenerating rat livers [28].

Hepatoma cell lines show system A transporters with altered sensitivity to sulfhydryl-modifying reagents [14,29], thus, we wondered whether the activity that develops during liver cell proliferation after partial hepatectomy could have any similarity with the one present in hepatoma cells. Sensitivity of system A to agents like NEM results in irreversible inactivation, which can be protected by the presence of system A substrate [14,29,30], thus supporting the idea that -SH groups are essential for substrate recognition and translocation. Altered sensitivity to NEM was consistent with carriers structurally different in hepatoma cells. Most hepatoma cell lines (HTC, H4, FAO, HepG2) show very low or nil sensitivity to this agent, but liver cells are highly sensitive [14]. Preincubation with NEM resulted in a concentration-dependent inhibition of Na^+ -dependent L-alanine transport, at a substrate concentration where system A contribution was predominant. In agreement with previous observations using plasma membrane vesicles [29], inhibition was maximal at 5 mM. A similar response was observed when studying R6 preparations and, thus, we think the activity which is up-regulated after partial hepatectomy does not share any similarity with the one found in hepatoma cell lines, and may be structurally similar or identical to the one present in non-induced cells. This feature is extremely interesting because it suggests that transformed cells may express transporters structurally different to the ones present in non-transformed cells and, at least theoretically, it opens the possibility of designing drugs able to selectively inhibit amino acid transporters of oncogenically transformed mammalian cells.

From all we have shown so far, we conclude that, early after hepatectomy, system A is up-regulated by a stable mechanism that does not involve changes in the Na^+ transmembrane gradient. The induced activity resembles basal system A and differs to the one expressed in hepatoma cell lines by its sensitivity to NEM treatment.

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